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# Expression of the dermonecrotic toxin by *Bordetella bronchiseptica* is not necessary for predisposing to infection with toxigenic *Pasteurella multocida*

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**Abstract**

This experiment was designed to determine whether a *Bordetella bronchiseptica* mutant that does not produce dermonecrotic toxin (DNT) is still capable of predisposing pigs to infection with toxigenic *Pasteurella multocida*. Three groups of pigs were initially inoculated intranasally with a wild type *B. bronchiseptica* that produces DNT, an isogenic mutant of *B. bronchiseptica* that does not produce DNT, or PBS. All pigs were then challenged intranasally with a toxigenic strain of *P. multocida* 4 days later. *P. multocida* was recovered infrequently and in low numbers from pigs initially inoculated with PBS, and no turbinate atrophy was present in these pigs. *P. multocida* was isolated in similar numbers from the pigs initially inoculated with either the wild type or the DNT mutant of *B. bronchiseptica*, and turbinate atrophy of a similar magnitude was also seen in pigs from both of these groups. Thus, although the DNT has been shown to be responsible for much of the pathology seen during infection with *B. bronchiseptica* by itself, infection with non-DNT-producing strains can still predispose to secondary respiratory infections with *P. multocida*. Published by Elsevier B.V.

*Keywords:* Atrophic rhinitis; *Bordetella bronchiseptica*; Dermonecrotic toxin (DNT); *Pasteurella multocida*

## 1. Introduction

Both *Bordetella bronchiseptica* and toxigenic strains of *Pasteurella multocida* cause atrophic rhinitis in pigs (de Jong, 1999). Infection with toxigenic strains of *P. multocida* (strains that produce the *P. multocida* toxin: PMT) result in a more severe and chronic form of the disease, referred to as progressive atrophic rhinitis, while infection with *B. bronchiseptica* alone is referred to as nonprogressive atrophic rhinitis. Infection with *B. bronchiseptica* predisposes pigs to infection with *P. multocida* (Chanter et al., 1989), and coinfection with these agents is often found in cases of progressive atrophic rhinitis.

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There are several potential mechanisms by which *B. bronchiseptica* predisposes to infection with other bacteria. Members of the genus *Bordetella* produce many virulence factors that are under the regulatory control of the *bvg* locus, a member of a broad family of two-component regulatory systems. One of these is the dermonecrotic toxin (DNT), an intracytoplasmic, heat-labile toxin. The DNT of *B. bronchiseptica* induces mucosal damage in swine nasal tissue and causes turbinate atrophy and pneumonic lesions characterized by necrosis, hemorrhage, neutrophil accumulation, and eventually fibrosis. Therefore, retarded clearance mechanisms, increased accumulation of mucus, exposure of submucosal areas where other bacteria may adhere, and increased nutrient availability could all play a role in increased colonization by other bacteria. Previous work demonstrated that DNT knockout mutants of *B. bronchiseptica* did not cause pneumonia or the turbinate atrophy characteristic of nonprogressive atrophic rhinitis, despite colonizing the respiratory tract well (Brockmeier et al., 2002). The experiment described here was designed to determine whether a DNT knockout mutant of *B. bronchiseptica* is still capable of predisposing pigs to infection with *P. multocida* and progressive atrophic rhinitis.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

*B. bronchiseptica* strain KM22, a virulent phase I isolate that produces DNT, and strain KB24, a *dnt* knockout mutant of KM22 (Brockmeier et al., 2002), were cultured on Bordet–Gengou agar supplemented with 10% sheep's blood (BG) at 37 °C for 40 h without or with the addition of gentamicin (100 μg/ml) to the medium, respectively. Suspensions of these cultures with an $A_{600}$ of 0.42 were prepared in phosphate buffered saline (PBS). This suspension has approximately $2 \times 10^9$ colony forming units (CFU)/ml, and a 1:2000 dilution of this suspension was made in PBS for inoculation of the pigs. Cultured dilutions of the strain KM22 and strain KB24 inocula each contained approximately $10^6$ CFU/ml. One hundred percent of the colonies of both strains appeared to be in the $Bvg^+$ phase, based on colony morphology and presence of hemolysis.

*P. multocida* strain 4533, a toxigenic type D isolate, was cultured on blood agar at 37 °C for 24 h. A suspension of this culture with an $A_{600}$ of 0.42 was prepared in PBS. This suspension has approximately $10^8$ colony forming units CFU/ml, and a 1:10 dilution of this suspension was made in PBS for inoculation of the pigs. Cultured dilutions of the strain 4533 inocula contained approximately $10^7$ CFU/ml.

### 2.2. Experimental infection in swine

Eighteen caesarian-derived, colostrum-deprived pigs were divided into three groups of six pigs each and inoculated intranasally at 1 week of age with 1 ml (0.5 ml/nostril) of a bacterial suspension of strain KM22, or its *dnt* knockout mutant strain KB24, or with 1 ml of sterile PBS. Tonsil and nasal swabs were obtained from all pigs prior to the start of the experiment, and no *B. bronchiseptica* or *P. multocida* was isolated. Four days after inoculation with *B. bronchiseptica* or PBS all pigs were inoculated with 1 ml (0.5 ml/nostril) of a bacterial suspension of toxigenic *P. multocida*. All housing, husbandry and experiments performed with pigs were in accordance with the law and approved by the Institutional Animal Care and Use Committee.

Small calcium alginate tipped swabs with 0.9 mm aluminum shafts that can be used for aural, nasal or urethral canals were used to swab the nasal cavity by inserting the swab approximately 6 cm into both nares. Nasal swabs, were taken from each pig 1, 2, and 3 weeks after inoculation with *P. multocida*, and the swabs were placed into tubes containing 500 μl PBS. Four weeks after inoculation with *P. multocida* the pigs were euthanized with an overdose of barbiturate, and necropsies were performed. Snouts were transected and removed at the level of the first premolar tooth and a 1 cm cross-section was cut from the caudal portion of the snout and used for atrophic rhinitis scoring. The entire right ventral turbinate from the remaining portion of the snout was removed for determination of the colonization of the turbinate. Subsequently, the tonsil was exposed, the full thickness of the entire right portion of the tonsil was removed, and an approximately 1 g full thickness piece from the center was removed for determination of colonization of the tonsil. The trachea was then severed just below the larynx and the trachea and lung were removed. A 1 cm

cross-section of trachea from the most cranial portion was removed and used for determination of colonization of the trachea. Finally, an approximately 1 g sample of lung was taken from the tip of the right cranial lung lobe for determination of colonization of the lung.

### 2.3. Determination of colonization

Serial 10-fold dilutions were made from the PBS solution in the tubes with the nasal swabs after vortexing the tubes with the swabs in them for 5 s. The number of CFU of *P. multocida* per ml was determined by plating 100 μl of the dilutions on duplicate selective blood agar plates containing 2 μg/ml amikacin, 4 μg/ml vancomycin, and 4 μg/ml amphotericin B. The number of CFU of *B. bronchiseptica* per ml was determined by plating 100 μl of the dilutions on duplicate selective blood agar plates containing 20 μg/ml penicillin, 10 μg/ml amphotericin B, 10 μg/ml streptomycin and 10 μg/ml spectinomycin. The lowest level of detection was 10 CFU/ml.

Four weeks after inoculation with *P. multocida*, when the pigs were euthanized, the specimens of nasal turbinate, tonsil, trachea and lung from each pig were weighed and ground individually in PBS to make a 10% weight:volume suspension. The number of CFU of *P. multocida* and *B. bronchiseptica* per gram of tissue was determined by plating 100 μl of the serial 10-fold dilutions of homogenates on duplicate selective blood agar plates as stated above. The lowest level of detection was 100 CFU/g of tissue.

### 2.4. Atrophic rhinitis scores

Snouts were transversely sectioned at the level of the first premolar tooth, and each of the four scrolls of the ventral turbinates was assigned an atrophy score that ranged from 0 to 4: 0 = normal, 1 = more than half of turbinate remaining, 2 = half or less of turbinate remaining, 3 = turbinate is straightened with only a small portion left, and 4 = total atrophy. The atrophic rhinitis score is the addition of the four turbinate atrophy scores and ranges from 0 to 16.

### 2.5. Statistics

A two-tailed, non-paired Student's *t*-test assuming unequal variance and a significance level of $P < 0.05$ was used to compare bacterial colonization levels and turbinate scores between the groups inoculated with strain KM22 ($DNT^+$) and strain KB24 ($DNT^-$) of *B. bronchiseptica*.

## 3. Results

The results of bacterial isolation from nasal swabs taken 1, 2, and 3 weeks after inoculation with *P. multocida* are given in Table 1. *B. bronchiseptica* was not isolated from the swabs at any of the time points from any of the pigs inoculated with PBS. *B. bronchiseptica* was isolated from the swabs at all of the time points from all of the pigs inoculated with either strain KM22 ($DNT^+$) or strain KB24 ($DNT^-$). Although there were, on average, slightly greater numbers of *B. bronchiseptica* isolated from the swabs of pigs inoculated with strain KM22 as compared to strain KB24, this difference was only statistically significant at week 2 ($P = 0.02$). Thus, both *B. bronchiseptica* strains were able to establish a comparable colonization level in the nasal cavity.

Table 1
Geometric mean ($\log_{10}$) numbers of *B. bronchiseptica* (Bb) and *P. multocida* (Pm) isolated from nasal swabs, and number of pigs from which the respective bacteria were isolated, after inoculation with PBS, *B. bronchiseptica* strain KB24 ($DNT^-$), or *B. bronchiseptica* strain KM22 ($DNT^+$) followed by toxigenic *P. multocida*

| | 1 week | | 2 weeks | | 3 weeks | |
|---|---|---|---|---|---|---|
| | Bb | Pm | Bb | Pm | Bb | Pm |
| KB24/Pm | 5.42 (6/6) | 4.23 (5/6) | 5.44 (6/6) | 3.87 (5/6) | 5.44 (6/6) | 3.55 (4/6) |
| KM22/Pm | 5.84 (6/6) | 4.54 (6/6) | 6.13* (6/6) | 5.82* (6/6) | 6.02 (6/6) | 5.12 (5/6) |
| PBS/Pm | NI (0/6) | 0.38 (1/6) | NI (0/6) | NI (0/6) | NI (0/6) | 1.10 (3/6) |

NI = not isolated.
* The difference in the level of colonization between groups inoculated with KM22 and KB24 was statistically significant; $P = 0.02$ for Bb at 2 weeks and $P = 0.03$ for Pm at 2 weeks.

Only small numbers of *P. multocida* were isolated from the nasal swabs of a few pigs in the group inoculated with PBS followed by *P. multocida*, whereas *P. multocida* was isolated from the nasal swabs of most pigs in both the groups that were inoculated with *B. bronchiseptica* followed by *P. multocida*. Similar to the results of *B. bronchiseptica* isolation, there were, on average, slightly greater numbers of *P. multocida* isolated from the swabs of pigs inoculated with strain KM22 ($DNT^+$) as compared to strain KB24 ($DNT^-$), but this difference was again only statistically significant at week 2 ($P = 0.03$). However, the mean number of *P. multocida* isolated from the pigs of both groups initially inoculated with *B. bronchiseptica* was significantly greater than the mean number isolated from pigs in the group initially inoculated with PBS ($P \leq 0.01$). Therefore, both $DNT^+$ and $DNT^-$ strains of *B. bronchiseptica* can enhance secondary infection of the nasal cavity with *P. multocida*.

The results of bacterial isolation from tissues taken at necropsy, 4 weeks after inoculation with *P. multocida*, are given in Table 2. At necropsy, *B. bronchiseptica* was not isolated from any of the tissues taken from pigs inoculated with PBS. *B. bronchiseptica* was isolated from all tissues from all pigs inoculated with either strain KM22 ($DNT^+$) or strain KB24 ($DNT^-$). Strain KM22 was isolated in greater numbers, on average, from the turbinate and trachea, and strain KB24 was isolated in greater numbers from the tonsil and lung. But the only significant differences in the number of *B. bronchiseptica* isolated from the two groups were significantly higher numbers of strain KM22 isolated from turbinates ($P = 0.01$) and significantly higher numbers of strain KB24 isolated from the tonsils ($P = 0.001$). Thus, again, both *B. bronchiseptica* strains were able to establish a comparable colonization level in tissues of the respiratory tract.

Table 2
Geometric mean ($\log_{10}$) numbers of *B. bronchiseptica* (Bb) and *P. multocida* (Pm) isolated from various tissues, and number of pigs from which the respective bacteria were isolated, after inoculation with PBS, *B. bronchiseptica* strain KB24 ($DNT^-$), or *B. bronchiseptica* strain KM22 ($DNT^+$) followed by toxigenic *P. multocida*

| Turbinate | | Tonsil | | Trachea | | Lung | | AR score |
|---|---|---|---|---|---|---|---|---|
| Bb | Pm | Bb | Pm | Bb | Pm | Bb | Pm | |
| KB24/Pm | | | | | | | | |
| 5.68 | 5.26 | 6.39* | 6.12 | 6.07 | 3.60 | 5.85 | 3.57 | 8.7 |
| 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 5/6 | 6/6 | 5/6 | |
| KM22/Pm | | | | | | | | |
| 8.00* | 5.80 | 4.54 | 6.02 | 6.76 | 4.14 | 5.16 | 2.98 | 10.7 |
| 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 4/6 | |
| PBS/Pm | | | | | | | | |
| NI | NI | NI | NI | NI | NI | NI | NI | 0.2 |
| 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | |

Atrophic rhinitis (AR) scores of pigs. NI = not isolated.
* The difference in the level of colonization between groups inoculated with KM22 and KB24 was statistically significant; $P = 0.01$ for Bb in turbinates and $P = 0.001$ for Bb in tonsil.

*P. multocida* was not isolated from any of the tissues taken from pigs inoculated with PBS followed by *P. multocida*. *P. multocida* was isolated from all the turbinates and tonsils, and from most of the tracheas and lungs of pigs inoculated with *B. bronchiseptica* followed by *P. multocida*. Although the same trend that was reported for isolation of *B. bronchiseptica* from the tissues occurred for *P. multocida* [*P. multocida* was isolated in greater numbers from the turbinate and trachea in pigs preinfected with strain KM22 ($DNT^+$), and was isolated in greater numbers from the tonsil and lung in pigs preinfected with strain KB24 ($DNT^-$)], none of these differences were statistically significant between groups inoculated with either strain KB24 or strain KM22. These results again demonstrate that both $DNT^+$ and $DNT^-$ strains of *B. bronchiseptica* can enhance secondary infection of the respiratory tract with *P. multocida*.

The atrophic rhinitis scores were in the normal range (0–1) for all the pigs inoculated with PBS followed by *P. multocida*. Atrophic rhinitis scores ranged from 2 to 14 for pigs inoculated with KB24 ($DNT^-$) followed by *P. multocida* and ranged from 6 to 14 for pigs inoculated with strain KM22 ($DNT^+$) followed by *P. multocida*. Although the mean score for the group inoculated with strain KM22 was slightly higher than that for the group inoculated with strain KB24, there was no significant difference between the mean atrophic rhinitis score for these two groups (Table 2).

## 4. Discussion

*P. multocida* was infrequently isolated in low numbers from pigs inoculated with PBS followed by *P.*

*multocida*, and no turbinate atrophy was present in these pigs. *P. multocida* was isolated in similar numbers from pigs initially inoculated with either strain KM22 ($DNT^+$) or strain KB24 ($DNT^-$) strains of *B. bronchiseptica*. Moderate to severe turbinate atrophy of a similar magnitude was also seen in pigs of both groups that received *B. bronchiseptica* prior to *P. multocida*. Thus, although the DNT of *B. bronchiseptica* is responsible for much of the pathology seen with infections of this organism alone (Brockmeier et al., 2002), infection with non-DNT-producing strains can still predispose to secondary respiratory infections with *P. multocida*.

Although the results reported here clearly demonstrate that non-DNT-producing strains of *B. bronchiseptica* can predispose to secondary infection with *P. multocida*, the results do not mean that DNT plays no role in this process. There are several previous studies that have examined the role of *B. bronchiseptica* DNT in predisposing to colonization with *P. multocida*. The first found that *P. multocida* colonized the nasal cavity in greatest numbers after preinfection with a DNT-producing strain of *B. bronchiseptica*, in intermediate numbers after preinfection with a non-DNT-producing strain, and lowest numbers after preinfection with a $Bvg^-$ strain (which would not produce DNT and a number of other virulence factors) (Chanter et al., 1989). In the above described experiment, colonization by the non-DNT-producing strain was not as great as the DNT-producing strain. This could have partially explained the difference in subsequent colonization by *P. multocida*. Additionally, the non-DNT-producing strain (PV6) was not a $DNT^-$ isogenic mutant of the DNT-producing strain (B58), thus, other differences between the strains could explain some of the differences in subsequent colonization with toxigenic *P. multocida*. For example, strain PV6 has a highly unusual PvuII ribotype (RT18) that is not typical of most swine *B. bronchiseptica* isolates like strain B58 and strain B65 (RT3) (Register et al., 1997; Register and Magyar, 1999). Strain PV6 also has unique pertactin and filamentous hemagglutinin types (virulence factors that are purported adhesins regulated by Bvg) that are not typical of other swine isolates (Register, 2001, 2003, 2004). For these reasons, strain PV6 may not be a good comparison to a typical virulent swine isolate (B58).

Another report describes preinfecting pigs with a non-DNT-producing strain of *B. bronchiseptica* (N-95), or pretreating the left nostril with purified *B. bronchiseptica* DNT, followed by inoculation with toxigenic *P. multocida* (Elias et al., 1992). *P. multocida* was subsequently only isolated from the left nostril and tonsil of pigs pretreated with purified *B. bronchiseptica* DNT. These results differ from our study and the study by Chanter, et al. where *P. multocida* was isolated from pigs preinfected with a non-DNT-producing strain. Again, the particular characteristics of strain N-95, including the fact it does not produce DNT, could have affected the results. The fact that pretreatment with purified *B. bronchiseptica* DNT resulted in subsequent *P. multocida* colonization makes the case that DNT can play a role in predisposing to secondary infections.

Finally, there is a report describing enhanced adherence of *P. multocida* to porcine tracheal rings preinfected with *B. bronchiseptica* (Dugal et al., 1992). The researchers ruled out DNT as the culprit through heat inactivation and size exclusion. Theorizing that the tracheal cytotoxin (TCT) was the culprit, purified TCT from *Bordetella pertussis* was also used to pretreat the tracheal rings, which resulted in enhanced colonization of *P. multocida*, as well. The TCT is a low molecular mass peptidoglycan fragment released from the cell wall of *Bordetella* species, and thus not under the control of Bvg, that appears to cause ciliostasis and damage to the respiratory epithelium. Thus, the above-mentioned study implicates not DNT, but TCT, in predisposing to secondary infection with *P. multocida*.

There are other mechanisms by which *B. bronchiseptica* may predispose to infection with other bacteria. These include specific interactions, such as piracy of adhesins, where organisms with limited capacity for colonization utilize secreted adhesins of other bacteria to promote attachment. *B. pertussis*, a highly related organism that causes whooping cough in humans, has been shown to enhance adhesion of secondary bacteria. *Streptococcus pneumoniae* and *Haemophilus influenzae* acquired the ability to adhere to cilia that were pretreated with filamentous hemagglutinin or pertussis toxin, two proteins that are secreted by *B. pertussis* and are known to mediate adherence to the cilia of respiratory epithelium (Tuomanen, 1986). *B. bronchiseptica* does not produce pertussis toxin but does produce filamentous hemagglutinin; thus, this could be a method by which

*B. bronchiseptica* increases adhesion of other bacteria in pigs. *B. bronchiseptica* is also cytotoxic for swine alveolar macrophages, which may result in decreased phagocytosis and clearance of bacteria in the lung (Brockmeier and Register, 2000). The adenylate cyclase toxin and type III secretion, both under the regulation of Bvg, have been implicated in *Bordetella*-induced cytotoxicity (Khelef et al., 1993; Stockbauer et al., 2003; Hewlett et al., 2006).

In conclusion, *B. bronchiseptica* may have redundant mechanisms by which it predisposes to *P. multocida*. Although *B. bronchiseptica* DNT may be able to enhance colonization with *P. multocida*, the results of this study clearly indicate it is not necessary. Other studies have shown that *B. bronchiseptica* can predispose to colonization with other bacteria such as *Haemophilus parasuis* and *Streptococcus suis* as well (Vecht et al., 1989; Brockmeier, 2004). It would be interesting to determine whether the same mechanisms predispose to secondary infection with these other bacteria. The results of this experiment are important when considering the development of attenuated intranasal vaccines or the possibility of using attenuated *B. bronchiseptica* strains as vectors for heterologous vaccines. Vaccines that may be attenuated due to the lack of DNT production could still contribute to potential secondary infections with other bacteria. More information delineating factors that contribute to the development of secondary infections needs to be gathered in order to make safe and efficacious vaccines.

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